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A Comparator Study with Observer-assisted Stereological Assessments

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ORIGINAL ARTICLE

Strong Prognostic Value of Tumor-infiltrating Neutrophils and Lymphocytes Assessed by Automated Digital Image Analysis in Early Stage Cervical Cancer: A Comparator Study with Observer-assisted Stereological Assessments

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ABSTRACT

INTRODUCTION

Manual observer-assisted stereological (OAS) assessments of tumor-infiltrating neutrophils and lymphocytes are prognostic, accurate, but cumbersome. We assessed the applicability of automated digital image analysis (DIA).

METHODS

Visiomorph software was used to obtain DIA densities of immunostains for $CD66b^+$ neutrophils, $CD163^+$ macrophages, and $CD8^+$ lymphocytes in tumors from 101 patients with International Federation of Gynecology and Obstetrics (FIGO) stage IB/IIA cervical cancer. Results were compared with manual OAS assessments.

RESULTS

Automated DIA assessment was faster and required less human resources than manual OAS assessments. We observed high correlations between DIA and OAS variables for $CD8^+$ lymphocytes, $CD66b^+$ neutrophils, and $CD163^+$ macrophages (spearman $\rho > 0.8$; $P < 0.0001$).

Hazard rates for recurrence of DIA assessments in the global tumor area were comparable with the prognostically strongest manual OAS assessments in the peritumoral compartment. In multivariate analysis, CD66b and CD8 densities, assessed by DIA, and regional lymph node metastases were independent predictors of RFS, while CD163 density and FIGO stage were not. The CD66b/CD8 tumor-associated neutrophil to lymphocyte (TA–NL) index accurately predicted the risk of relapse, ranging from 8% to 52% ($P = 0.001$).

CONCLUSIONS

DIA is a potential assessment technique. The TA–NL index obtained by DIA is a strong prognostic variable with possible routine clinical application.

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Collection and assembly of data: AC, BSN, HH, and PSN collected the data
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Cervical cancer remains a cause of morbidity and mortality in women even with the introduction of screening and vaccine programs. Prognostic markers for patient outcome in localized cervical cancer consist of clinicopathological features although tissue biomarkers are emerging.[1,2] The introduction of new prognostic markers is warranted for improved patient care. Immune infiltrates have emerged as potential prognostic factors in a range of cancers.[3] In previously published studies by our group in patients with early stage cervical cancer we demonstrated that high densities of tumor-infiltrating lymphocytes were associated with a favorable recurrence-free survival (RFS) whereas high densities of tumor-associated CD66b$^+$ neutrophils were associated with a poor PFS.[4–6] These results were obtained by an observer-assisted stereological (OAS) method that is accurate and reproducible[7] but labor intensive and requires specialized training.[8,9] For clinical relevance and applicability more efficient methods are warranted.

Digital image analysis (DIA) is an emerging, high-throughput method for automated quantitative assessments of immunostained sections. Software for automated DIA of whole slide images has evolved rapidly and allows for fast quantification of the distribution of immunohistochemical (IHC)-stained cells or structures within a large area in less time and with reduced workload compared to observer-assisted methods.[9] Care has to be taken as DIA protocols are still sensitive to variation by tissue processing, IHC protocols, unspecific staining, and definition of region of interest (ROI).[8,10,11]

In the present study, we developed automated DIA protocols for quantifying CD66b neutrophils, CD163 macrophage, and CD8 lymphocyte immunostains in the same cohort of early stage cervical cancer patients as previously assessed with the OAS method. We found automated DIA provided clinically applicable prognostic information comparable to the considerably more cumbersome manual OAS methods in a fast, efficient, and robust manner.

MATERIALS AND METHODS

The study included 101 consecutive patients treated with surgery ($N = 87$) or definitive radiotherapy ($N = 14$) for cervical squamous cell type carcinoma of International Federation of Gynecology and Obstetrics (FIGO)[12] stage IB ($N = 91$) and IIA ($N = 10$) at Aalborg Hospital, Denmark, from 1990 to 2000.[4] Clinical subclassification in stage IB1 and IB2 was not available. Mean age was 44 years (22–70 years). If operable, patients underwent hysterectomy with pelvic lymph node dissection and in cases of increased risk of relapse, radiotherapy was added. Inoperable patients were treated by definitive radiotherapy (combined external radiotherapy and brachytherapy). At the time of surgery 18 patients had lymph node metastases. Adjuvant radiotherapy was given to 23 patients. Median follow-up was 9.8 years; for patients alive minimum follow-up was at least 5 years. During the follow-up period a total of 31 patients relapsed. Tumor samples were collected from the blocks used for routine pathologic evaluation. If patients were treated with definitive radiotherapy biopsy material was collected, otherwise whole tumor blocks were retrieved. We aimed at analyzing two tumor sections from each patient; however, in 16 patients (16%) only one section was available.

The study was approved by the local Ethics Committee (Case number M-20100011; date 9 February 2010).

IMMUNOHISTOCHEMISTRY

Formalin-fixed, paraffin-embedded tumor specimens were sectioned at 2 μm and mounted on glass slides. Primary antibodies were against CD66b (clone G10F5, 1:600, no. 555723, BD Biosciences, United States), CD163 (clone EDHu-1, 1:100, MCA 1853, Abd Serotec, United Kingdom), and CD8 (Clone C8/144B, 1:250, M 7103, Dako). Immunohistochemistry was performed using a Benchmark XT automated stainer (Ventana Medical Systems, Tucson, AZ, United States). Sections were counterstained with haematoxylin and bluing reagent. The IHC protocols have previously been described in detail.[4,5]

QUANTITATIVE EVALUATION OF IMMUNOSTAINING

Whole slide images were captured by NanoZoomer 2.0 (Hamamatsu Photonics K.K., Hamamatsu City, Japan) using the 20× magnification mode. The global tumor area including the tumor area and adjacent tumor-associated stroma defined at low screen magnification, excluding areas of large necroses was outlined as ROI by the observer (Fig. 1A). Generally necrotic areas were scarce and in almost all cases no adjustment for necroses was performed. In four sections, some necrotic areas were observed with a degree of peri-necrotic infiltration of $CD66b^+$ neutrophils. However, in theses sections the global tumor area was highly infiltrated by $CD66b^+$ neutrophils and consequently a correction for peri-necrotic infiltration was not deemed necessary. We did observe CD66b IHC staining of the necrotic areas but little CD163 staining.

For DIA, three automated protocols were developed using Visiomorph DP software (Visiopharm, Denmark) for assessing the densities of CD66b (Fig. 1B), CD163, and CD8 immunostains, respectively (i.e. the area of immunostain divided by the tissue area within the ROI).

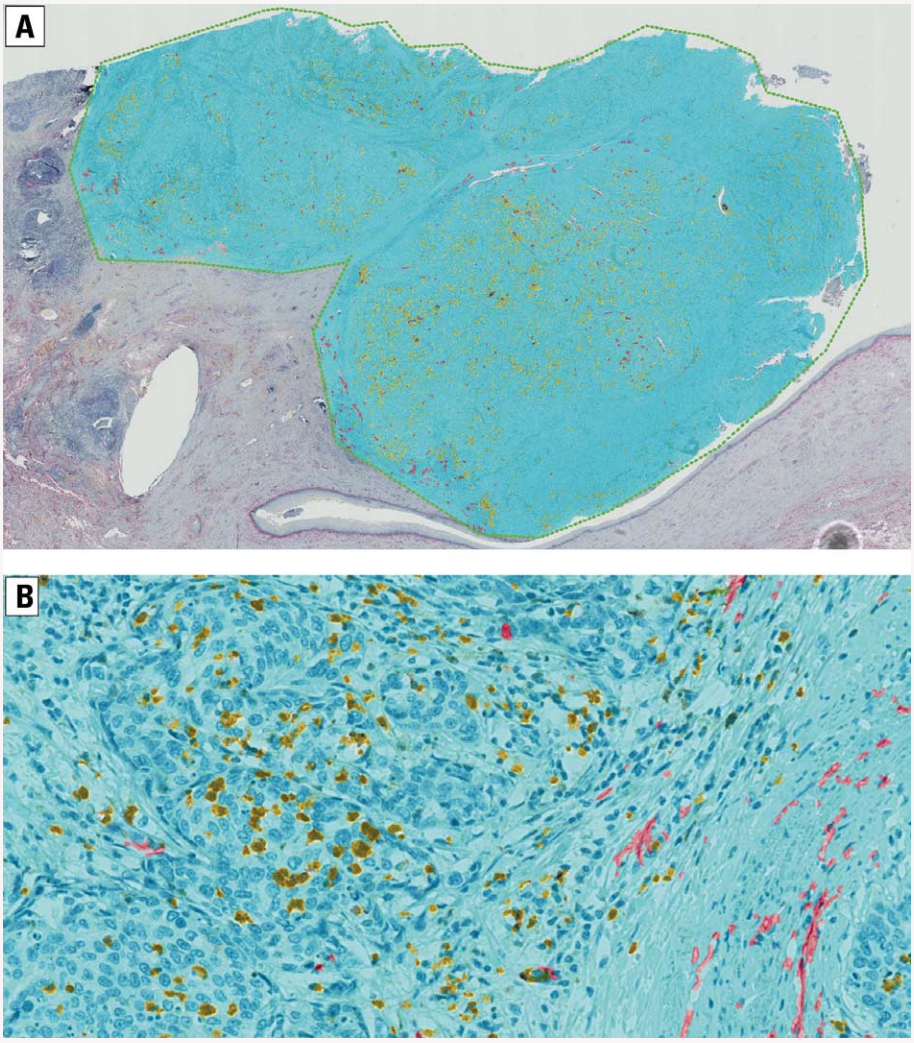

Figure 1. (A) The Digital Image Analysis (DIA) Allows for Discrimination between Tissue (Blue Area) and Irrelevant Space within the Region of Interest (ROI) (Yellow Line) (resolution 20x). (B) DIA Determines the Density of Immunostain (Orange) Relative to Tissue (resolution 400x).

The protocols were devised by assessing 8–10 representative sections and encompassed a series of preprocessing steps (enhancement of red–green–blue color levels and, if relevant, Diaminobenzidine (DAB) deconvolution), segmentation (Bayesian classifier), and post-processing steps (mainly morphological operations).

OAS assessments of immune cells in three, nonoverlapping tumor compartments (tumor nests, peritumoral and stromal compartments, respectively), were obtained using computer-aided, unbiased stereological sampling techniques (newCAST software, Visiopharm, Denmark), as described in detail previously.[4] An area of stroma was denoted peritumoral, if at least one malignant cell was observed in the field of view inside a sampling frame. If no tumor cells were observed the area was denoted as stromal. The OAS assessment of $CD163^+$ macrophages was performed by point counting yielding a density of $CD163^+$ immunostain per area of tissue, as macrophages are often elongated and fragmented rendering individual cell delineation impossible. In contrast, OAS assessments of $CD66b^+$ neutrophil and $CD8^+$ lymphocyte were performed as cell profile counts per area of tissue. For comparison with DIA, results of OAS variables obtained in the global tumor area were calculated from the sum of estimates in all compartments of the tumor, weighted according to sampling intensity.

STATISTICS

The DIA assessments of CD66b, CD163, and CD8 immunostains were positively skewed and some assessments had a value of 0. Therefore, log-transformation after adding a constant of 1 was applied to achieve approximate normality.[13] Nonparametric spearman rank test was performed to investigate correlations between non-transformed DIA assessments and OAS assessments. Pearson correlation tests were also performed on the log-transformed values but yielded similar results (data not shown). A linear regression analysis was performed to compare log-transformed values of OAS and DIA assessments of $CD163^+$ macrophage immunostain. A nonparametric Mann–Whitney test was performed to compare variables obtained in patients with and without lymph node metastases. For prognostic analyses, variables were dichotomized at medians or quartiles. Univariate Cox regression models were used to assess the hazard ratio (HR) of the DIA and OAS variables. OAS assessments performed in the peritumoral compartment, which in our previous study generally had the highest discriminatory power,[4] were included in the prognostic models for comparison. A Cox proportional hazards model was created to identify independent predictors of RFS, including DIA immunostain densities of $CD66b^+$ neutrophils, $CD163^+$ macrophages, and $CD8^+$ lymphocytes, as well as clinical prognostic variables.

Statistical analyses were performed using SPSS version 20.0 (SPSS, Chicago, IL, United States) statistical software. All tests were two-sided and P values less than .05 were considered statistically significant.

RESULTS

The patient demographics and clinicopathological features are summarized in Table 1.

In univariate analysis increased infiltration of $CD66b^+$ neutrophils (HR 2.9; 95% CI 1.3–6.3; $P = 0.007$) and low densities of $CD8^+$ lymphocytes (HR 2.4; 95% CI 1.1–5.1; $P = 0.02$) assessed by automated DIA were significantly associated with poor RFS. Increased infiltration of $CD163^+$ macrophages was not significant ($P = 0.06$) in univariate analysis. These results obtained by DIA were comparable to previously obtained OAS measurement results assessed in

Table 1. Patient Characteristics ($N = 101$).

	Without recurrence	Recurrence
Number of patients	70 (69%)	31 (31%)
Age at diagnosis (range), years	45 (26–68)	41 (22–70)
Stage IB	63 (90%)	28 (91%)
Stage IIA	7 (10%)	3 (9%)
Lymph node metastases		
No	62 (89%)	21 (68%)
Yes	8 (11%)	10 (32%)
Primary treatment		
Surgery	61 (87%)	26 (85%)
+Adjuvant radiotherapy	13 (21%)	10 (38%)
Radiotherapy	9 (13%)	5 (15%)

the peritumoral compartment[4] (shown for comparison in Table 2).

For an experienced observer, time spent to obtain OAS densities of immune cells by manual cell counting, using a sampling frame with associated sampling points in automatically selected fields of visions, was approximately 2 min for delineation of the sampling area and roughly 15 min per cell variable per patient. In contrast, total observer time spend for DIA was only approximately 2 min per patient, used for delineation of the ROI. Computation runtime for a single slide was 2–15 min, and the total analysis of an unlimited number of slides could be performed at any time, day or night, with no request of observer assistance.

We observed high correlations between DIA and OAS variables of corresponding parameters (Fig. 2A–C). For $CD8^+$ lymphocytes spearman ρ was 0.79 (95% CI 0.68–0.87; $P < 0.0001$); for $CD66b^+$ neutrophils spearman ρ was 0.85 (95% CI 0.75–0.91; $P < 0.0001$); and for $CD163^+$ macrophages spearman ρ was 0.92 (95% CI 0.88–0.95; $P < 0.0001$). The $CD8^+$ lymphocyte and $CD66b^+$ neutrophil assessments obtained by the two methods are not directly comparable, since the DIA yields an immunostain area fraction, whereas the OAS counts produces a numerical density of immunostained cells per tissue area.[4] Hence, the correlation between these variables is not expected to be linear, best illustrated in Figure 2A for neutrophils. Linear regression analysis of the similar DIA-obtained and OAS-obtained $CD163^+$ macrophage immunostain densities revealed an excellent correlation ($R^2 = 0.86$; $P < 0.0001$; Fig. 2C).

A multivariate Cox proportional-hazard regression model was used to analyze the relative strength of DIA assessments of $CD66b^+$ neutrophils, $CD163^+$ macrophages, and $CD8^+$ lymphocytes in addition to FIGO stage and lymph node status. High density of CD66b immunostain (HR 2.6; 95% CI 1.2–5.7; $P = 0.02$), low density of CD8 immunostain (HR 2.3; 95% CI 1.1–4.9; $P = 0.03$), and presence of lymph node metastases (HR 2.6; 95% CI 1.2–5.5; $P = 0.02$) were significant independent factors associated with of poor RFS, whereas clinical stage and CD163 immunostain density were not.

The CD66b/CD8 tumor-associated neutrophil to lymphocyte (TA–NL) index obtained by DIA had excellent discriminatory power for each quartile with 5-year RFS of 92%, 80%, 65%, and 48% for quartile I (<0.019), II (0.02–0.05), III (0.06–0.24), and IV (>0.25), respectively ($P = 0.001$; Fig. 3).

The DIA $CD163^+$ macrophage immunostain density was significantly lower in patients with lymph node metastases (median 0.9% (95% CI 0.6–1.6%)) compared to those without (median 2.0% (95% CI 0.9–3.3%)) ($P = 0.007$), but the two groups CIs were overlapping. No significant correlation among $CD66b^+$ and $CD8^+$ immunostain densities and lymph node metastases was observed.

DISCUSSION

In the present study, we demonstrated assessment of a CD66b/CD8 TA–NL index obtained by automated DIA accurately predicted the risk of relapse for early stage cervical cancer patients. Automated DIA is a fast and efficient method compared to manual assessments and has potential for routine application. This study is the first direct comparison between an automated and manual IHC assessment in cervical cancer and validates our previous findings of the prognostic impact of tumor-associated immune cells.[3–5]

Table 2. Univariate Comparison of Prognostic Impact with Respect to Recurrence-Free Survival (RFS) of Cervical Carcinoma, for Digital Image Analysis (DIA) Assessments in the Global Tumor Area and for Previously Obtained Observer-Assisted Stereological (OAS) Assessments in the Peritumoral Compartment. $CD66b^+$ Neutrophils, $CD163^+$ Macrophages, and $CD8^+$ Lymphocytes, Stratified at Median.

Risk factor	Median cutoff	DIA (HR)	OAS (HR)
$CD66b^+$	> vs. ≤	2.9 (1.3–6.3; $P = 0.007$)	2.1 (1.0–4.5; $P = 0.04$)
$CD8^+$	≤ vs. >	2.4 (1.1–5.1; $P = 0.02$)	3.4 (1.5–7.7; $P = 0.003$)
$CD163^+$	> vs. ≤	2.0 (0.97–4.2; $P = 0.06$)	2.1 (1.0–4.4; $P = 0.05$)

Hazard ratio (HR) for RFS (95% CI; *P* value); vs. = versus.

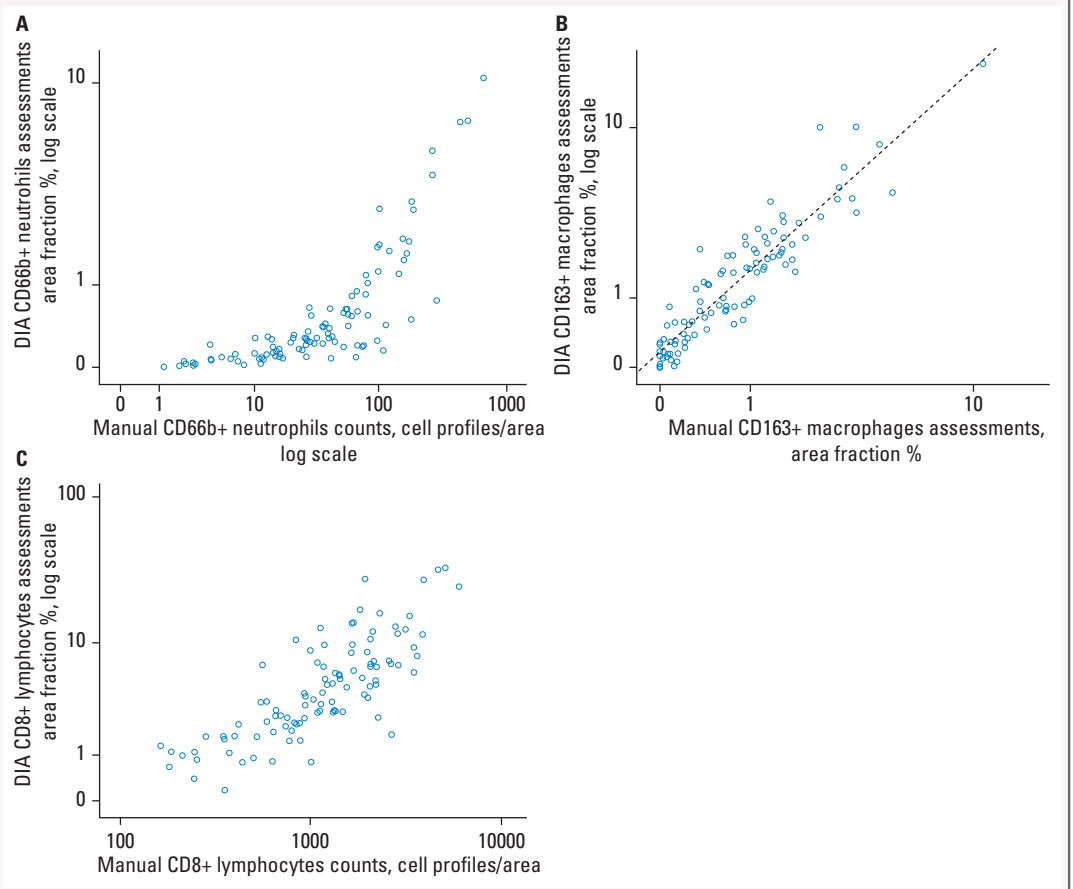

Figure 2. **Scatterplots of log + 1-transformed Values for Immunostain Density Assessments Performed by Automated DIA in the Global Tumor Area (y Axis) Versus Corresponding Variables Obtained by Observer-assisted Stereology (OAS) in the Peritumoral Compartment (x Axis).** (A) $CD66b^+$ Immunostain Density vs. Numerical Density of $CD66b^+$ Neutrophils. (B) $CD163^+$ Macrophage Immunostain Densities Obtained by DIA vs. OAS. (C) $CD8^+$ Immunostain Density vs. Numerical Density of $CD8^+$ Lymphocytes.

The prognostic importance of the tumor immune infiltrate has emerged in many cancer types. High numbers of tumor-associated $CD66b^+$ neutrophils and $CD163^+$ M2 macrophages have been correlated with poor outcome in several malignant diseases,[14–16] although some results have been conflicting.[17,18] Neutrophils are involved in crucial steps for cancer development, including increased carcinogenesis, initiation of the angiogenic switch, extravasation of tumor cells, and formation of the "premetastatic niche".[14] In murine studies tumor-associated neutrophils (TAN) have been observed to polarize into N1 neutrophils with antitumor activity and N2 neutrophils with tumor-promoting properties.[19] If polarizations of TANs occur in human tumors are unclear. The tumor sections in the present study were reviewed by a senior pathologist (T.S.) and close to all cells with morphological resemblance of segmented neutrophils stained positively for CD66b. No significant morphological heterogeneity of neutrophils was observed. CD66b is a marker of neutrophil activation, but whether CD66b stain all TANs or only a subset of TANs is unknown. The number of tumor-promoting neutrophils relative to tumor-antigen-responsive mononuclear cells may predict patient outcome as suggested by studies of these cells in the peripheral circulation of cancer patients.[14,16,20] Lymphocytes infiltrating cervical cancers and other gynecological cancers have been recognized as prognostically favorable for decades,[21–24] and the efficacy of the host immune response to

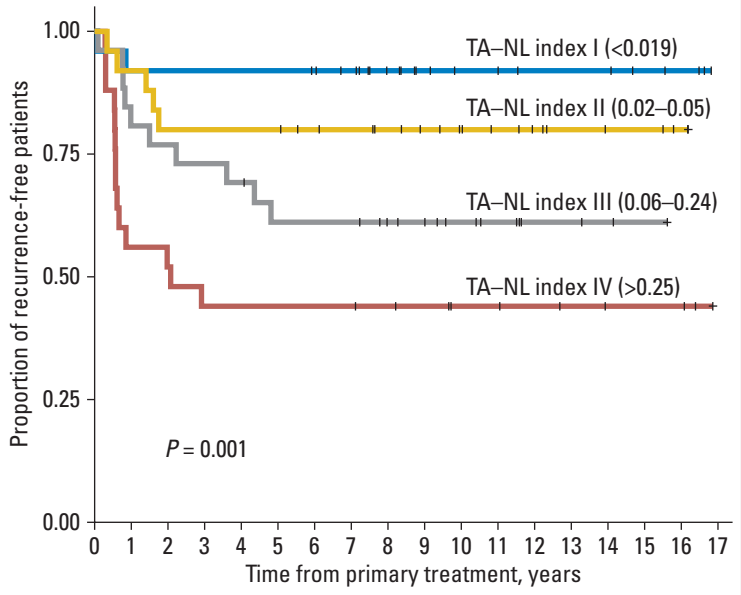

Figure 3. **Kaplan–Meier Plots of RFS of 101 Patients with Localized Cervical Squamous Cell Carcinoma According to the CD66b/CD8 Immunostain (TA–NL) Index in the Global Tumor Area Divided at Quartiles. *P* value Obtained from Log-Rank Test.**

virus-associated antigens and oncogenes seems reflected by the composition, localization, and numbers of specific types of tumor-infiltrating lymphocytes *in situ*. For example, in CIN 2–3 lesions HPV-16 infection correlated with low numbers of $CD8^+$ lymphocytes, and the number of stromal $CD8^+$ lymphocyte was an independent regression predictor.[25] We demonstrated in previous studies of manual stereological assessments of $CD8^+$ lymphocytes and $CD66b^+$ neutrophils excellent prognostic information of the composition of the immune infiltrate with some tumor compartment variation. In this novel study of automated DIA analysis in the global tumor compartment, we demonstrated a prognostic impact of a tumor-associated CD66b/CD8 immunostain index with possible clinical applicability. This could identify high-risk patients, who may benefit the most from adjuvant treatment.[26,27]

In recent years it has become increasingly clear that in addition to cancer cells, a tumor lesion contains a number of recruited normal cells that contribute to the hallmarks of cancer by creating the tumor microenvironment.[28] Stromal cells, blood vessels and infiltrating inflammatory cells are major components of the tumor microenvironment.[29] These cells enable and sustain most of the hallmarks of cancer through reciprocal communications with neoplastic cancer cells.[30] A leukocyte infiltrate, comprising mast cells, T cells, natural killer cells, T-regulatory cells, myeloid-derived suppressor cells, tumor-associated macrophages, and TANs are key participants of the tumor microenvironment where they can promote or inhibit cancer formation and development.[31–33] The novelty of the present study is to provide a fast, efficient assessment method to identify key prognostic components of the tumor microenvironment in cervical cancer making this information potentially clinically translatable. Modification of the tumor microenvironment may be a target for therapeutics.[34,35]

Variables obtained by DIA in the global tumor area and by the OAS technique in the peritumoral compartment had similar power to identify subgroups of patients with poor and favorable prognosis. OAS assessments of tumor-associated leukocytes obtained at the "invasion front", that is, in the peritumoral compartment was the prognostically most informative in our OAS assessments. In contrast, a simple DIA observation obtained in the global tumor compartment provided similar prognostic information. However, for routine application in a clinical setting

the global tumor area is rapidly defined with ease and therefore DIA has potential for routine use. Software solutions associating immunostains to individual cells of specific morphological types are in some instances available,[9] but the robustness of automated morphological cell identification is debatable and must be validated for specific cell types and protocols. Double staining methods may be applicable in some instances as have been shown in malignant melanoma for DIA assessments of the frequency of Ki67 and phosphohistone expression in MART1(Melan-A)-positive tumor cells.[36]

Even for an experienced observer the observer time consumption was reduced substantially with DIA compared with a manual observer-assisted method (OAS) method. Variables obtained by DIA and by the OAS techniques had similar ability to identify subgroups of patients with poor and favorable prognosis. In particular, the CD66b/CD8 TA–NL index obtained by DIA had excellent discriminatory power, identifying precisely the risk of cancer recurrence in each quartile of the index. This immune cell index seems highly suited for routine application, robust to heterogeneity and automatically obtainable after quick and simple outlining of an easily definable ROI. It is obtainable by a trained technician and, potentially, in a single double-immunostained section. Still, DIA protocols may be sensitive to variations in tissue processing and staining protocols,[8,10,11] and the interlaboratory reproducibility of measurements should therefore be investigated further.

A limitation to our study was the retrospective design and heterogeneity of treatments and amount of tumor tissue available for analysis. However, patients were treated consecutively in a single institution. We did not observe any impact on our results of treatment modality or whether one or two tumor sections were available.

In early stage cervix cancer additional prognostic factors are needed to identify high-risk patients. The results of the CD66b/CD8 TA–NL index obtained by DIA are promising and should be validated prospectively in future studies.

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest. AC had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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